Inhibition of GABA transporters fails to afford significant protection following focal cerebral ischemia

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Abstract
Brain ischemia triggers excitotoxicity and cell death, yet no neuroprotective drugs have made it to the clinic. While enhancing GABAergic signaling to counterbalance excitotoxicity has shown promise in animal models, clinical studies have failed. Blockade of GABA transporters (GATs) offers an indirect approach to increase GABA inhibition to lower the excitation threshold of neurons. Among the GATs, GAT1 is known to promote neuroprotection, while the protective role of the extrasynaptic transporters GAT3 and BGT1 is elusive. A focal lesion was induced in the motor cortex in two to four-month-old C57BL/6J male mice by photothrombosis. The GAT1 inhibitor, tiagabine (1 and 10 mg/kg), the GAT2/3 inhibitor, (S)-SNAP-5114 (5 and 30 mg/kg) and the GAT1/BGT1 inhibitor, EF-1502 (1 and 10 mg/kg) were given i.p. 1 and 6 h post-stroke to assess their impact on infarct volume and motor performance seven days post-stroke. One mg/kg tiagabine improved motor performance, while 10 mg/kg tiagabine, (S)-SNAP-5114 and EF-1502 had no effect. None of the compounds affected infarct volume. Interestingly, treatment with tiagabine induced seizures and (S)-SNAP-5114 led to increased mortality. Although we show that tiagabine can promote protection, our findings indicate that caution should be had when using GAT1 and GAT3 inhibitors for conditions of brain ischemia.

Introduction

Stroke is the second leading cause of death and long-term disability worldwide, resulting in impaired quality of living and increased societal and economic burden. The mechanisms underlying the deleterious effects of an ischemic stroke are well documented and include glutamate-mediated excitotoxicity and extensive cell death.[1,2] Of the many compounds trialed, thrombolytic agents remain the only currently approved pharmacological treatment, although they have to be given within 4.5 h of stroke onset, and are only effective in 5–10% of all patients.[3] However, recent data from the DAWN Trial on endovascular clot retrieval, presented at the European Stroke Organization Conference (ESOC), May 2017, demonstrate a 73% reduction in disability in stroke patients receiving treatment up to 24 h post-stroke. This compelling data shows an extended therapeutic window does exist in humans and that there still remains an urgent need to discover new therapies that can minimize the extent of cell death or that can be given as adjuncts to further enhance recovery.

γ-Aminobutyric acid (GABA), the main inhibitory neurotransmitter in the mammalian brain, is initially released in the acute phase of stroke, in response to

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impaired ATP production.[4] The released GABA acts on both synaptic and extrasynaptic $GABA_A$ receptors, mediating phasic and tonic inhibition, respectively.[5] Termination of $GABA_A$ receptor-mediated signaling is further regulated by GABA uptake through GABA transporters (GATs), present on both presynaptic neuronal membranes and astrocytes.[4] In particular, GAT3, which is preferentially found on astrocytes at extrasynaptic sites,[6] has been found to play a role in stroke pathogenesis, as it is down-regulated after stroke, causing GABA accumulation and enhanced tonic inhibition, while GAT1 is unaffected in terms of level and function.[7] The delayed increase in tonic inhibition has been proposed to be an endogenous protective mechanism, although the increase occurs at such a delay that it is unlikely to have much of an effect on cell death.[7–9] Accordingly, enhancing synaptic GABAergic signaling acutely (within hours) has been shown convincingly to be neuroprotective in animal stroke models.[2] However, compounds that preferentially target synaptic GABA receptors have failed to translate into humans,[10] most likely due to the instability of synaptic $GABA_A$ receptors, in the presence of high GABA concentrations.[11]

With extrasynaptic $GABA_A$ receptors and tonic inhibition being implicated in the regulation of brain excitability, and recently in stroke recovery,[7,8,12,13] interest has emerged in understanding the role of tonic GABA-mediated inhibition in the acute phase of stroke and neuroprotection. One potential mechanism to achieve this is by inhibition of GATs, thereby increasing ambient GABA levels and tonic inhibition.[12] Four GATs have been identified (GAT1-3 and BGT1),[14] which are proposed to be differently involved in the regulation of GABA signaling, owing to their different cellular distribution and molecular pharmacology.[4,15] Blockade of GAT1, which is expressed strongly on presynaptic neurons, but also on glial cells, has been shown to promote neuroprotection post-stroke in rats and gerbils.[16–19] However, the role of GAT1 in mice as well as pharmacological blockade of the glial GABA transporter GAT3[6,7] and the scarcely brain-expressed transporter, BGT1,[20] in relation to neuroprotection, is yet to be investigated. Given the co-localization of GAT3 and BGT1 with extrasynaptic $GABA_A$ receptors, and their postulated role in mediating tonic inhibition,[7,21,22] they have both been proposed as candidate targets in neuroprotection.[4,7,20,23] Further, although BGT1 is poorly expressed in the brain, it has been shown that BGT1 may be a target for modulating brain excitability.[24,25] In addition, the expression of BGT1 has been shown to increase in response to several pathological conditions,[23,26] although much work is still required to ascertain the exact physiological role of BGT1 in the brain.

Therefore, the overall aim of the present study was to determine the neuroprotective potential of blocking

Table 1. Inhibitory activities of GAT inhibitors used in this study.

Ligand	IC$_{50}$ values (µM)			
	GAT1	GAT2	GAT3	BGT1
Tiagabine	0.8	>300	>800	>300
(S)-SNAP-5114	388	21	5	140
(R,S)-EF-1502	7	>300	>300	26

Note: Data obtained from [^{3}H]GABA uptake experiments using recombinantly expressed mouse GATs.[29] GAT: GABA transporter.

GAT1, GAT3 and BGT1, using the photothrombotic (PT) stroke model to introduce a focal lesion in the motor cortex of mice. The PT model produces a small focal infarct that rapidly evolves to be fully formed by about 24 h, accompanied only by a low degree of collateral reperfusion, limiting the over-interpretation of neuroprotective effects.[9,27,28] To investigate the neuroprotective potential of GATs after a PT stroke, we used three currently available inhibitors (Table 1); tiagabine which is a selective GAT1 inhibitor,[29] (S)-SNAP-5114 which is a GAT2/3 inhibitor,[30] and (R,S)-EF-1502 which is a GAT1/BGT1 inhibitor.[31] This is the first study to assess in parallel the role of GAT1, GAT2/3 and GAT1/BGT1 blockade in neuroprotection in mice subjected to a PT stroke.

Methods

Animals

Young (two to four-month-old) C57BL/6J male mice (Hercus Taieri Research Unit, Dunedin, New Zealand) weighing 24–30 g were used. They were housed in groups of three to five mice under conditions of 12 h light/dark cycle (light off at 6 a.m.) with free access to water and food. All experiments were approved by the University of Otago Animal Ethics Committee and carried out in accordance with the NIH Animal Protection Guidelines for the care and use of animals for scientific purposes and reported according to the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines. The animals were acclimatized for at least seven days prior to the experiments.

Photothrombosis model of focal ischemia

Focal stroke was induced in the mice (73 in total) as previously reported,[7] before the mice were randomly assigned into a treatment group. Under isoflurane anesthesia (2–2.5% in O_2), mice were placed in a stereotactic apparatus. A cold light source (KL1500 LCD, Zeiss) attached to a 40× objective giving a 2 mm

diameter illumination was positioned 1.5 mm lateral from Bregma at the exposed intact skull, and 0.2 mL Rose Bengal solution (10 g/L in normal saline, i.p.: Sigma-Aldrich, St. Louis, MO, USA) was administered. After 5 min, the brain was illuminated for 15 min, while keeping body temperature at $37.0 \pm 0.3°C$ by a heating pad (Harvard apparatus, Holliston, MA, USA). The mice were returned to their home cage after a short recovery period and held under normal housing conditions.

Compound administration

Tiagabine and (S)-SNAP-5114 were purchased from Sigma-Aldrich. (R,S)-EF-1502 (EF-1502) was produced in house as previously described.[31] Tiagabine (1 mg/kg and 10 mg/kg) and EF-1502 (1 mg/kg and 5 mg/kg) were dissolved in sterile isotonic saline with 2% DMSO. (S)-SNAP-5114 (5 mg/kg and 30 mg/kg) was dissolved in sterile isotonic saline with 10% DMSO due to its low solubility. All animals were randomly assigned to a treatment group post-stroke, to ensure that all animals in any given cage received a different treatment. The compounds were administered at a low and high dose based on previously published EC_{50} values obtained in other disease models with no notation of seizure-inducing effects.[24,32,33] While control animals were injected with 2% DMSO in isotonic saline to serve as controls for tiagabine and EF-1502, 10% DMSO in isotonic saline was injected to serve as controls for (S)-SNAP-5114. Each compound or vehicle were administered i.p. (12 µL/g of body mass) 1 and 6 h post-stroke, as all compounds have been shown to modulate brain excitability after i.p. administration at doses in the same range as used in the present study.[32,34] Mice were then returned to their home cage after each injection and observed for 5-10 min per hour for the first 2 h post-compound administration for signs of abnormal behaviors.

Infarct size

The mice were deeply anesthetized with pentobarbital (University of Otago, Animal Welfare Office, New Zealand) and transcardially perfused with 4% paraformaldehyde (PFA) seven days post-stroke. The brains were removed and post-fixed for 1 h in 4% PFA before being transferred to 30% sucrose. The brains were cut on a sliding freezing stage microtome in six coronal parallel sets in sections of 40 µm thickness and kept in cryoprotectant at $-20°C$. Infarct volume was determined by histological assessment using cresyl violet staining according to a previously published protocol.[7] Infarct volume was quantified using Image J (National Institutes of Health, USA) by an observer blind as to the treatment groups, and is based on obtaining measurements from every 6th section through the entire infarct (area in mm^2), and infarct volume was quantified as follows: infarct volume $mm^3 = \sqrt{area\ mm^2} \times section\ thickness \times section\ interval$. A total of six mice had stroke volumes less than $0.6\ mm^3$ and were subsequently excluded on the basis that the stroke induction was incomplete (1 from the 10 mg/kg tiagabine group; 3 from the 5 mg /kg EF-1502 group; 2 from the 10% DMSO vehicle group). One and five mice from the 5 mg/kg and 30 mg/kg (S)-SNAP-5114 groups, respectively, were found dead the day after the compound had been administered, and therefore the final number of mice for infarct and behavioral analysis were: 2% DMSO vehicle, $n = 10$; 10% DMSO vehicle, $n = 8$; 1 mg/kg tiagabine, $n = 6$; 10 mg/kg tiagabine, $n = 8$; 1 mg/kg EF-1502, $n = 8$; 10 mg/kg EF-1502, $n = 8$; 5 mg/kg (S)-SNAP-5114 $n = 7$; 30 mg/kg (S)-SNAP-5114, $n = 6$.

Behavioral assessment

Recovery of forelimb motor function was determined by the cylinder and grid-walking tasks as previously reported by observers blind as to the treatment groups.[7] The mice were tested once in each task approximately seven days prior to stroke to establish a baseline level, and then seven days post-stroke. The resulting behavior was scored as previously described by an observer blind as to the treatment groups.[7]

Statistical analysis

All statistical analysis was carried out using GraphPad Prism version 7.0 b (GraphPad Software, San Diego, CA, USA). One-way ANOVA followed by Dunnett's post hoc analysis was used to test for differences in infarct volume. Two-way ANOVA followed by Dunnett's post hoc analysis was used to test for differences in motor function with time and treatment as independent factors and time as repeated measures. $p < 0.05$ was considered statistically significant.

Group sizes were determined by power analysis calculations based on previous data obtained in the cylinder and grid-walking tasks[7,27] with the following parameters: $\alpha = 0.05$; with an effect size $= 1.5$. Using six animals per group, we thus obtain a power of 83% for behavioral experiments. The power calculation was performed with G Power Software (version 3.1.5).

Results

Blockade of GATs has no effect on infarct volume

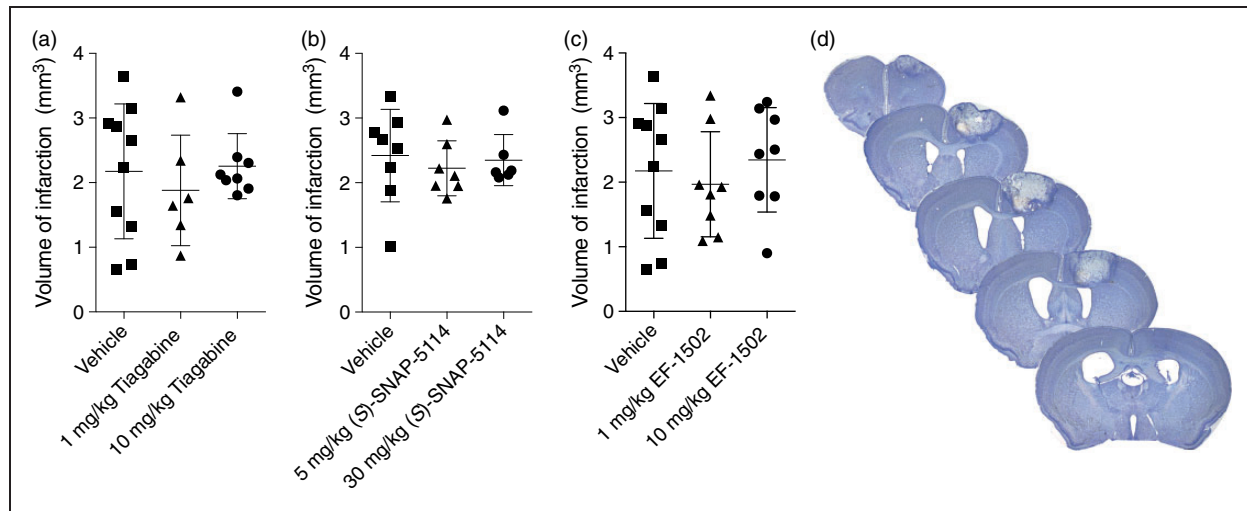As many strokes lack a reperfusion component, we decided to investigate the protective effects of tiagabine

Figure 1. Infarct volume seven days post PT-induced stroke. (a–c) No significant difference was found between treatment groups (one-way ANOVA) (d) Representative cresyl violet stainings of brain slices, seven days post-stroke, from an animal treated with the vehicle with 2% DMSO. Data is presented as mean $\pm$ SD.

(GAT1 inhibitor), EF-1502 (GAT1/BGT1 inhibitor) and (S)-SNAP-5114 (GAT2/3 inhibitor) in the focal PT model of stroke. This stroke model is characterized by minimal reperfusion and a rapidly evolving core, and hence a model in which the extent of cell death is much harder to minimize within the therapeutic window of around 6 h.[35]

Mice were treated with an initial i.p. dose of tiagabine (1 or 10 mg/kg), EF-1502 (1 or 5 mg/kg), (S)-SNAP-5114 (5 or 30 mg/kg) or vehicle (2% or 10% DMSO in saline) 1 h post-stroke, with a second dose given at 6 h. Infarct volumes were assessed seven days post-stroke using cresyl violet staining and ranged from 2.18 to 2.42 mm^3 in vehicle-treated animals, which is in line with previous reports.[35] No differences were observed between the 2% or 10% DMSO-injected control groups. Acute blockade of GAT1 by tiagabine ($p > 0.73$), GAT1/BGT1 by EF-1502 ($p > 0.85$) and GAT2/3 by (S)-SNAP-5114 ($p > 0.72$) had no effect on infarct volume, at either low or high doses, when measured seven days post-stroke (Figure 1).

Blockade of GATs has no effect on motor function

We next tested the mice behaviorally on both the grid-walking (forelimb function) and cylinder (forelimb asymmetry) tasks. Behavioral assessments revealed a significant increase in the number of foot faults on the grid-walking task and in spontaneous forelimb asymmetry in the cylinder task seven days post-stroke ($p < 0.0001$; Figure 2). Acute treatment with 1 mg/kg tiagabine significantly decreased forelimb asymmetry in the cylinder task (Figure 2(a)), but had no effect on the number of foot faults in the grid-walking task

(Figure 2(b)). Treatment with 10 mg/kg tiagabine, however, had no effect on either forelimb asymmetry or number of foot faults (Figure 2(a) and (b)). Also, treatment with either (S)-SNAP-5114 or EF-1502 had no effect on forelimb asymmetry (Figure 2(c) and (e)) or the number of foot faults (Figure 2(d) and (f)) at either of the doses tested.

Seizure-like behavior after tiagabine treatment

Several of the mice treated with tiagabine suffered from seizures following either a single 1 h dose or both doses (1 and 6 h) of tiagabine. One out of six mice from the 1 mg/kg treatment group and five out of eight mice from the 10 mg/kg treatment group exhibited increased shaking in the paws including some forelimb clonus, hunched posture including piloerection and head tics, all signs of low-grade seizure activity. Even though these mice exhibited low-grade seizures, they were still included in the analysis of infarct volume and motor function. No animals in the EF-1502 and (S)-SNAP-5114 groups displayed any obvious signs of seizure-like behavior. However, five out of 11 mice treated with the high dose of (S)-SNAP-5114, and one out of nine treated with the low dose of (S)-SNAP-5114, were found dead the following day. Even though no signs of gross seizure activity were observed, low grade or absence seizures, or even cardiovascular complications cannot be ruled out.

Discussion

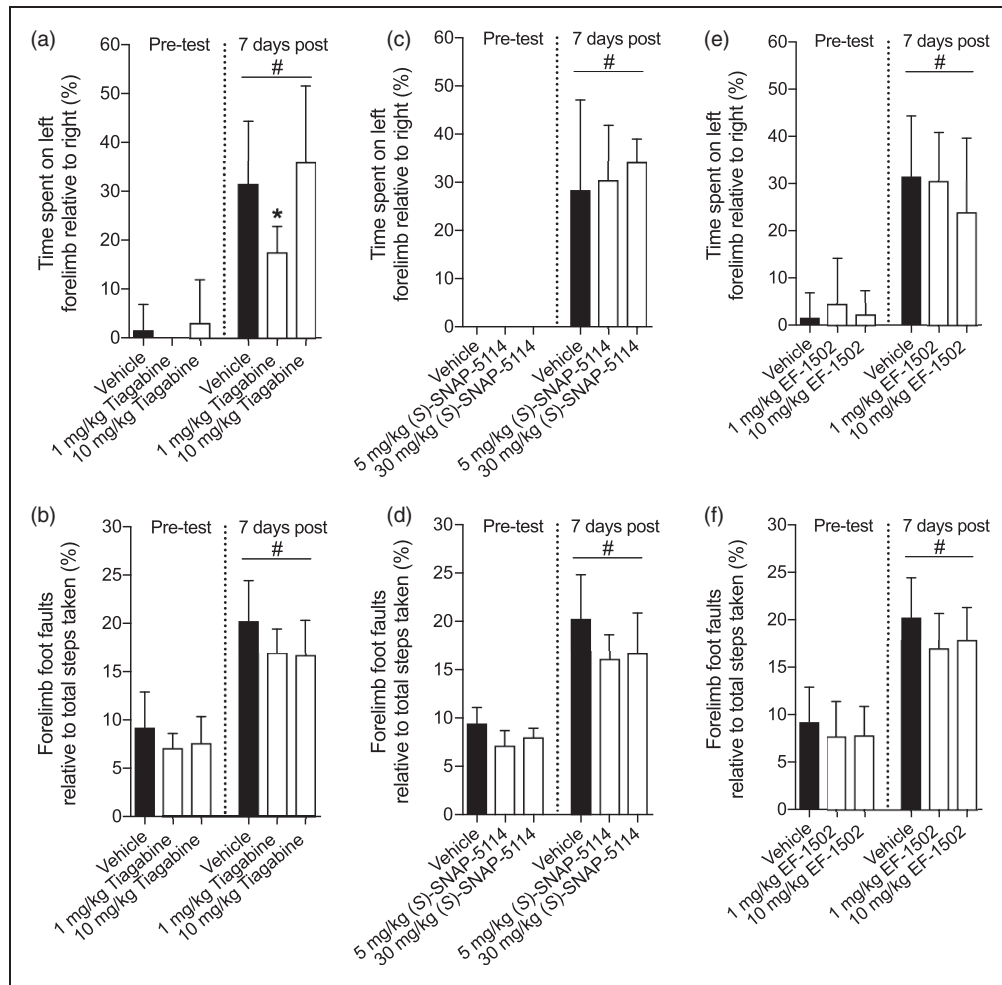The renewed interest in modulating GABA levels after an ischemic stroke, not only deals with the role of

Figure 2. Motor function assessment seven days post PT-induced stroke. Motor function was assessed with forelimb asymmetry and number of foot faults in the cylinder and grid-walking tasks, respectively. Stroke decreased the forelimb asymmetry and increased the number of foot faults seven days post-stroke ($\#p < 0.0001$ pre-test vehicle compared to vehicle seven days post-stroke). (a) Two-way ANOVA followed by Dunnett's multiple comparison test with time and treatment as independent factors revealed that treatment with 1 mg/kg tiagabine but not 10 mg/kg tiagabine decreased forelimb asymmetry seven days post-stroke in the cylinder task compared to vehicle treated stroke animals (1 mg/kg tiagabine: $p = 0.037$, 10 mg/kg tiagabine: $p = 0.6217$; time: $F_{(1, 21)} = 66.43$, $p < 0.0001$; treatment: $F_{(2, 21)} = 3.188$, $p = 0.062$; interaction: $F_{(2, 21)} = 1.811$, $p = 0.188$). (b) Assessment of tiagabine treatment on the grid-walking task showed no differences on the number of foot faults at either 1 or 10 mg/kg. (c, d) (S)-SNAP-5114 (e, f) or EF-1502 had no effect on forelimb asymmetry or number of foot faults seven days post-stroke at either dose tested. Data are presented as mean $\pm$ SD (2% DMSO vehicle, $n = 10$; 10% DMSO vehicle, $n = 8$; 1 mg/kg tiagabine, $n = 6$; 10 mg/kg tiagabine, $n = 8$; 1 mg/kg EF-1502, $n = 8$; 10 mg/kg EF-1502, $n = 8$; 5 mg/kg (S)-SNAP-5114, $n = 7$; 30 mg/kg (S)-SNAP-5114, $n = 6$).

GABA$_A$ receptor subtype-selective modulation and tonic inhibition, but also with indirect modulation, i.e. through the involvement of the GATs.[7] The delayed increase in tonic inhibition after stroke is mediated in part by an impairment in GAT3 function.[7] This increase in tonic inhibition is thought to be an endogenous neuroprotective mechanism; however, as it remains elevated, it also hinders functional recovery after stroke.[7,36] The GATs are differently involved in the regulation of GABAergic signaling due to differences in localization and expression levels, and the development of subtype-selective ligands for the GATs could

be relevant for future drug discovery.[4,12] GAT1 controls both phasic and tonic inhibition, while GAT3, and presumably BGT1 control tonic inhibition based on their localization close to extrasynaptic GABA$_A$ receptors.[7,21,22,24,37] We are the first to perform parallel studies to assess if acute blockade of GAT1 by tiagabine, GAT2/3 by (S)-SNAP-5114 and GAT1/BGT1 by EF-1502 promotes neuroprotection after a focal model of stroke in mice.

Prior studies using rats and gerbils have shown that tiagabine reduces the infarct size in animals subjected to common carotid artery occlusion-induced

ischemia.[16–19] We are the first to show that 1 mg/kg tiagabine improves motor performance (forelimb asymmetry) in mice subjected to a PT-induced stroke, while 10 mg/kg tiagabine had no effect on motor function. In contrast to previous findings in rats and gerbils, tiagabine had no effect on the infarct volume in mice. Both differences in the species, and the dose and model used could influence this. For instance, the positive effect of tiagabine on infarct volume in rats and gerbils was observed at doses in the same range or higher than the ones used in the present study, and may contribute to the divergent findings.[16–19] However, based on our finding of increased seizure activity, increasing the dose is not an option. Also, there are obvious differences in the stroke outcome produced by occlusion and PT, e.g. due to differences in reperfusion, which is missing after a PT stroke, extent of oxidative damage and size of the penumbra, which are smaller after PT making it harder to obtain protection.[28] Even though the infarct volumes produced by PT are smaller than the ones produced by artery occlusion,[28] infarct volumes of the given size, which are comparable to the size of human strokes, have been shown to be susceptible to neuroprotective agents.[35] Also, it has recently been shown that anesthetics, including isoflurane which is used in the present study, can mask the full neuroprotective effects of therapeutic compounds.[38] As a result, future studies are still required to ascertain to what extent tiagabine and other GAT inhibitors can afford protection in awake freely moving mice.

Consistent with previously reported findings,[16–19] we also observed seizures in some of the tiagabine-treated animals after stroke. From clinical use of tiagabine and from GAT1 knock-out mice, it is clear that impaired GAT1 uptake can induce seizures as well as non-convulsive status epilepticus in humans, through an enhancement of tonic inhibition rather than depletion of neuronal GABA.[39] We have recently observed that GAT3 levels decrease very early after stroke, within 6 h, (unpublished data) which is much earlier than first reported.[7] Prior studies have reported that GABA uptake in the sensorimotor cortex is synergistically regulated by both GAT1 and GAT2/3 function.[7,40] Since tonic inhibition is increased already after one day[41] and GAT3 is impaired from quite early on after a PT stroke,[7] one likely explanation for the stroke-induced seizures observed, is that blocking GAT1, even with low doses of tiagabine, could enhance tonic inhibition to detrimental levels. As not all animals in our study and not all studies have reported increased seizure activity with tiagabine post-stroke,[16,19] it is likely that in animals that showed no seizure activity upon tiagabine treatment that GAT3 is still functional at the time of compound administration. GAT3 expression may then decrease at a later time point, or not at all in other species or other stroke models, and therefore can still contribute to the maintenance of cortical excitability. This is supported by our recent study showing that GAT3 expression in the peri-infarct is unaffected 1 and 3 h post-stroke but lowered from 6 h post-stroke, (unpublished data) clearly illustrating that GAT3 expression undergoes drastic changes in the time window by which the compounds were administered. Nevertheless, GAT3 should still be susceptible for further inhibition within 6 h post-stroke according to the temporal expression profile of GAT3. Further, since we observed protection with the low dose of tiagabine, it is possible that it is better to target GAT1 with low doses, e.g. IC_{10} rather than IC_{50}, to promote protection, e.g. to counterbalance excitotoxicity and without triggering tonic GABA-induced seizures.

In our studies, we did not see any effect of (S)-SNAP-5114 in respect to infarct volume and motor function seven days post-stroke. Although we have shown that GAT3 function is impaired seven days post-stroke in untreated mice,[7] while GAT3 expression is decreased 6 h post-stroke in the peri-infarct, it still remains to be investigated if GAT3 function is impaired within hours after the insult, where (S)-SNAP-5114 was administered. One possible explanation for the incurred mortality observed in response to (S)-SNAP-5114 could be a (S)-SNAP-5114-induced enhancement of tonic inhibition via GATs, causing absence or silent seizures,[42] e.g. via GAT3 inhibition distal to the infarct, but it could also be related to general toxicity of the compound or its metabolites in the brain or in the periphery as GAT3 is expressed in many different tissues including the heart, pancreas and kidneys. Although (S)-SNAP-5114 is the best GAT3 inhibitor commercially available in terms of selectivity, the limited brain uptake of (S)-SNAP-5114, lack of subtype-selectivity together with its low solubility, and poor chemical stability,[43,44] limits its usability to study the role of GAT3 specifically in acute stroke in humans.

The low potency of EF-1502 at GAT1 compared to tiagabine, together with the low brain expression of BGT1, may explain why EF-1502 did not affect infarct volume and motor function at the doses tested, and did not display seizure-inducing activity. Differences in the potency of EF-1502 and tiagabine have been reported previously in a mouse model of epilepsy, showing that the anti-epileptic potency of EF-1502 is approximately 10-fold lower than tiagabine.[33]

Conclusion

These studies show that inhibition of the GATs in the acute phase of a focal ischemic stroke has limited neuroprotective potential when the inhibitors are

administered acutely, 1 and 6 h after stroke. Although it is feasible to promote protection via inhibition of GAT1, the translational potential of tiagabine in stroke patients is limited due to the induction of post-stroke seizures, presumably mediated by excess tonic inhibition. To investigate the neuroprotective potential of only inhibiting GAT3 or BGT1 in acute stroke, better subtype-selective, brain-permeable and potent inhibitors for the two transporters are needed.[4] Finally, based on the unexplained deaths using the GAT3 inhibitor, (*S*)-SNAP-5114, caution should be had in terms of both novel drug development and clinical testing for condition of brain injury with GAT inhibitors. Given the discrepancies between different animal stroke models and species, much further work is required to assess and correlate IC_{10} and IC_{50} concentrations to changes in tonic inhibition and neuroprotection where GATs may or may not be already impaired post-stroke.

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Declaration of conflicting interests

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Authors' contributions

MEKL, ANC and PW designed the studies. RPC provided (*R,S*)-EF-1502 and assisted with chemical expertise. MEKL, EKG and ANC performed and analyzed the in vivo studies. MEKL drafted the manuscript. PW and ANC critically revised the manuscript. All authors reviewed, edited and approved the final version of this manuscript.

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